IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: CARL-HENRIK HELDIN et al.

Serial No.: 041,299 Group Art Unit: 185

Filed: 22 April 1987 Examiner: Carson

For: RECOMBINANT DNA ENCODING PDGF A-CHAIN POLYPEPTIDES

RULE 131 DECLARATION

The Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

6/5/89
Robert L. Ref
6/5/89

Sir:

I, GRAEME I. BELL, HEREBY DECLARE:

1. I am a true, named coninventor of subject matter disclosed and claimed in the subject application.

2. The invention(s) claimed in the subject application were reduced to practice in the United States before 13 August 1986. The foregoing statement is based upon the following facts of which I have personal knowledge.

3. At least two PDGF A-chain cDNA clones were reduced to practice in the United States before 13 August 1986. Exhibit A, submitted herewith, is a true copy of Betsholtz et al., Nature, Vol. 320, pp. 695-699 (24 April 1986), which discloses cDNA clone D1 of human PDGF A-chain in Figure 1 and at page 695. This is the same clone D1 disclosed in the subject application (see, e.g.,

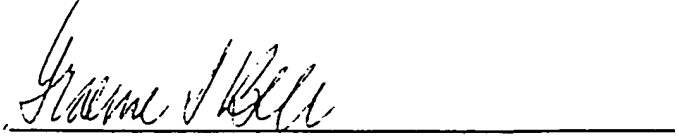
Figure 1 of the application). Coinventors Heldin, Betsholtz, Westermark, Knott and Scott are coauthors of this paper. As shown at page 699 of Betsholtz et al., the manuscript was received by Nature on 24 January 1986, thus proving that clone D1 was made before 13 August 1986. The clone D1 was received in the laboratories of Chiron Corporation, located in Emeryville, California, where I was employed at the time the invention(s) were made, before 13 August 1986.

4. Exhibit B, submitted herewith, consists of pages from the laboratory notebook of N. Fong, who was employed by Chiron Corporation in Emeryville, California at the time the invention(s) were made. Exhibit B is a true copy of the notebook pages with the exception of the indicated dates, which have been blacked out. The pages of Exhibit B, however, are dated before 13 August 1986. Exhibit B shows that N. Fong was provided two clones of human PDGF A-chain, D1 and 13-1, for isolation of the coding sequence and subsequent DNA sequencing. These are the same clones called D1 and 13-1 in the subject application, for example, in application Figures 1 and 2.

5. Exhibit C also consists of copies of pages from the laboratory notebook of N. Fong. Exhibit C is a true copy of these pages with the exception of the indicated dates, which have been blacked out. These pages, however, are

dated before 13 August 1986. Exhibit C describes the ligation of PDGF A-chain coding sequences from clones D1 and 13-1 into a pSV7d expression vector, as also described in Example 2 (pp. 18-23) of the subject application. More specifically, Exhibit C shows the successful ligation of the coding sequence from clone D1 into the pSV7d expression vector, the resulting vector being called "phPDGFA-103". This is the same or equivalent to vector "pSV7d-PDGF-A103" described in Example 2 of the subject application.

6. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By 

Graeme I. Bell

Date May 31, 1989

From Page No.

DATE: [redacted]

GIVEN: 2 clones containing the human platelet-derived growth factor A-chain cDNA

"D1" plasmid ~1300-1500bp (EcoRI)

"13-1" λ clone slightly smaller than 1300 bp (EcoRI)

PLAN: ISOLATE EcoRI FRAGMENTS FROM EACH OF THESE CLONES FOR SEQUENCING

"D1" PLASMID "pUC13-D1 1mg/ml"

V_T = 500λ

Mix: 200λ - EcoRI mix *
100λ - DNA in TE (100μg)
180λ - H_2O
20λ - EcoRI (New England Biolabs) 10 units/λ

React: 37°C, 12:55pm -

Test: 2λ + 3λ - H_2O + 2λ - EtOH stop
on Δ gel (1% Agarose)

* FINAL BUFFER: 100 mM Tris, pH 7.5 / 50mM NaCl / 5 mM $MgCl_2$ / 100μg/ml BSA / 0.1% β-mercaptoEtOH

To Page No.

Witnessed & Understood by me, Date

Invented by [redacted] Date [redacted]

Exhibit B
1 of 2

TITLE — Project No. ______

Book N ______

From Page No. ___

"13-1" λ Clone

V_T = 200λ

mix: 80.0λ – EcoRI mix
~~30λ~~ ~~37.5λ~~ – DNA in TE (15µg)
~~85.0λ~~ ~~77.5λ~~ – H_2O
5.0λ – EcoRI (NEB, lot 17, 20 U/λ)

react: 37°C, 12:55pm -

TEST: 9λ + 2λ - ~~STOP~~ Ficoll on A gel

both Digestions complete

PREPS → "D1 PLASMID"

~~85~~ ~~50.0λ~~ 2+50λ – TE
(15.0λ – Ficoll)

λ 13-1

(50 λ – TE
(15 λ – Ficoll)

Store @ -20°C [mixture]

prep gel: 1% Agarose
135 mA

cut out FRAGS → cut and pass through
in ID-salt elutip-D buffer { 16 GA, 18 GA, 20 GA, 22 GA } needles rt, o/n.

To Page No. 22

Witnessed & Understood by ______

Invented by [illegible signature]

Recorded by

Date [redacted]

Exhibit B
2 of 2

LIGATION TO expression VECTOR.

VECTOR: pSV7d /ECORI / -PO4
[redacted] LR
CST. 100 ng/λ 2.423 kb
Dilute 2λ into 8λ-H2O

DNA: DI - 1300 bp 4 ng/λ
λ13-1 - 600 bp ≤ 20 ng/9λ
λ13-1 - 1300 bp ≤ 20 ng/9λ

Cf: 4 μg/ml

FRAGMENT: VECTOR RATIO 2:1

λ13-1 // 600bp Cf = 2 μg/λ → 20.0λ

MIX: 9.0λ - 600 bp - λ13-1 DNA
2.0λ - 20 ng/λ VECTOR
2.0λ - 10x KIN. B.
2.0λ - 10 mM ATP
4.0λ - H2O
+ 1.0λ - T4 Lig (NEB, 400U/λ lot 26)

λ13-1 // 1300bp Cf = 2 μg/λ → 20.0λ

MIX: 9.0λ - 1300 bp - λ12-1 DNA
2.0λ - 20 ng/λ VECTOR
2.0λ - 10x KIN. B.
2.0λ - 10 mM ATP
5.0λ - H2O
+ 1.5λ - T4 LIG. (")

Exhibit C
1 f 4

Witnessed & Understood by me. Date Invented by [redacted] Date [redacted]
Recorded by

28

Project No.

Book No. TITLE hPDGF-a-chain

From Page No.

D1 (1300) /Vector

~~[illegible]~~

ζ_f = 3 µg/ml V_f = 20λ

8λ - DNA (32 ng)
1.5λ - Vector (30 ng)
2.0λ - 10 mM ATP
2.0λ - 10x lig. B
5.0λ - H_2O
+ 1.5λ - T4 lig (11)

React: 14°C, o/n   [Initials [redacted]]

Date: [redacted]

~~[illegible]~~ prod → 92.5λ - H_2O

Add: 7.5λ - 1M $CaCl_2$ (fresh)
200.0λ - Competent HB101 E. coli

60' @ 4°C
5' @ 42°C

Add: 3.0 ml - Luria Broth
37°C, 1 hr w/ agitation air shaker

Exhibit C
2 of 4

Witnessed & Understood by me, Date Invented by [redacted] Recorded by

screening transformants
phPDGFΔ-102

Project No.______
Book No.______ TITLE phPDGFΔ-102

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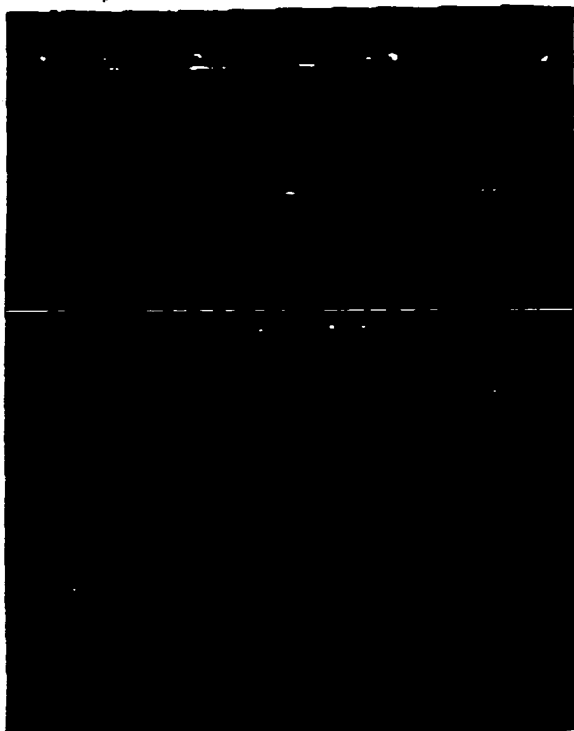
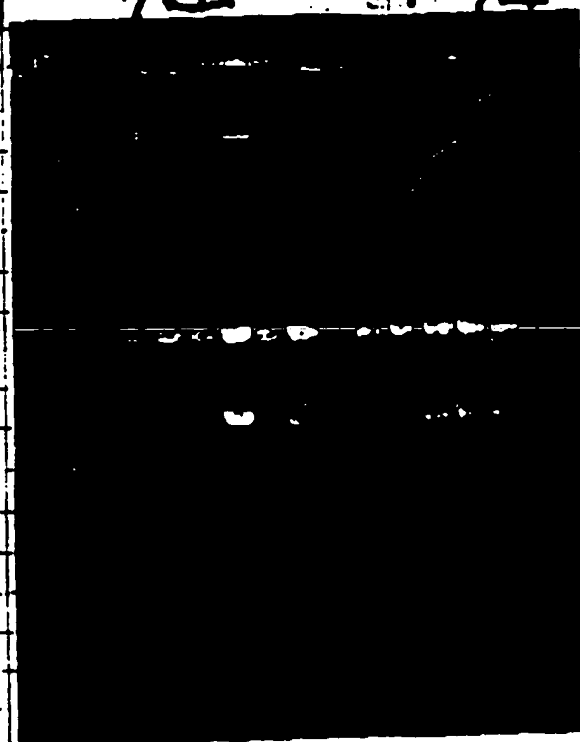
From Page No.___

phPDGFΔ-102
7/13-1 [illegible]
Bam, HindIII



phPDGFΔ-102
CDT/RI + [illegible] plasmid
/RI



Update [redacted]

gels run by Anne

phPDGFΔ-102 (7/13-1/RI → 1800 + 600)

Attempt to clone this

no clones.

To Page

Witnessed & Understood by me, | Date | Invented by | Date | Recorded by | Date of [redacted]

Exhibit C
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TITLE

From Page No. 26

WAIT FOR MORE DNA (GBC1).
~~MORE INTO VECTOR~~
CUT W/ ECORI
LIGATE MIXTURE INTO THE VECTOR.

pHPDGFA-103 ~~USABLE CLONES~~: (D1/E1+ pSV7d)
CORRECT CLONES: 2, 5, 7
WE STILL DON'T KNOW ORIENTATION.
CUT W/ BAMHI + HindIII.
(WILL BE DONE BY ANNE O.)

D1

pUC 13 - PLASMID INTO HB101

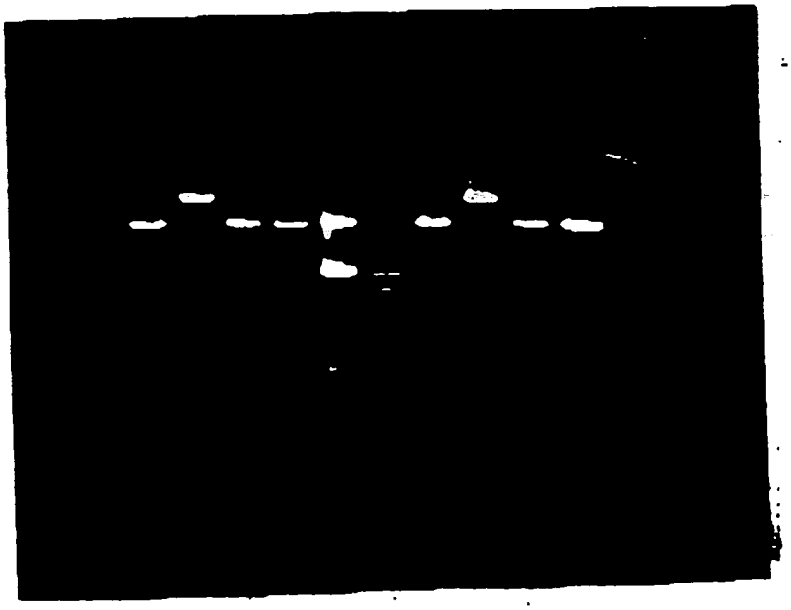
WE GOT IT → GLYCEROL STOCK MADE.

These clones
cut w/
BamHI +
HindIII →

#2, #7
are the
correct
orientation

MAKE GLYCEROL
STOCKS OF
BOTH

→ LB + AMP
PLATES



To Page No.

Witnessed & Understood by
Date

Exhibit C
4 of 4

cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines

Christer Betsholtz*, Ann Johnsson†, Carl-Henrik Heldin†**, Bengt Westermark*, Peter Lind‡**, Mickey S. Urdea§, Roger Eddy‖, Thomas B. Shows‖, Karen Philpott¶, Andrew L. Mellor¶, Timothy J. Knott* & James Scott*

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The amino-acid sequence of the precursor of the human tumour cell line-derived platelet-derived growth factor (PDGF) A-chain has been deduced from complementary DNA clones and the gene localized to chromosome 7. The protein shows extensive homology to the PDGF B-chain precursor. Expression of the PDGF A-chain gene is independent of that of the PDGF B-chain in a number of human tumour cell lines, and secretion of a PDGF-like growth factor of relative molecular mass 31,000 correlates with expression of A- but not B-chain messenger RNA.

HUMAN platelet-derived growth factor (PDGF) consists of dimers of homologous polypeptide chains, denoted A and B (refs 1, 2). Whether PDGF is a heterodimer or a mixture of homodimers is not known, but the dimer structure is functionally important, since reduction irreversibly destroys the biological activity of PDGF. Connective tissue-derived cells display high-affinity cell-surface receptors for PDGF and respond to PDGF by receptor autophosphorylation, tyrosine phosphorylation of cytoplasmic substrates, increased cytoplasmic calcium concentration, activation of protein kinase C, cytoplasmic alkalinization, reorganization of actin filaments, specific gene expression and DNA synthesis (reviewed in ref. 3).

The B-chain precursor is encoded by the c-*sis* gene, the cellular counterpart to the transforming gene v-*sis* of simian sarcoma virus (SSV)[2,4-6]. The human c-*sis* gene has been mapped to the long arm of chromosome 22 (ref. 7) and has been shown to be transcribed in several human tumour cell lines[8-11] as well as in certain normal cell types such as endothelial cells[12], placental cytotrophoblasts[13] and activated macrophages[14,15].

The primary translation product of the v-*sis* gene undergoes dimerization and proteolytic processing at the N- and C-terminals, yielding a product of relative molecular mass (M_r) 24,000 (24K) which resembles a dimer of PDGF B-chains and is recognized by anti-PDGF antibodies[16]. There is ample evidence that SSV-induced transformation is mediated by a PDGF-like growth factor. First, SSV-transformed cells contain and release a PDGF agonist activity[17-22]. Second, acutely SSV-transformed human fibroblasts are morphologically indistinguishable from PDGF-stimulated cells, and more significantly, their transformed phenotype is reverted by the addition of anti-PDGF antibodies to the culture medium[23]. Studies of the transforming protein of SSV have indicated that assembled PDGF B-chains alone form an active mitogen. Furthermore, amino-acid sequence analysis of porcine PDGF has revealed that this dimeric factor contains only one type of chain, corresponding to the human B-chain[24].

Evidence that homodimers of PDGF A-chains also have biological activity was recently obtained from studies of a PDGF-like mitogen released from a human osteosarcoma cell line, U-2 OS. This factor[25], which binds to the PDGF receptor, was found to be a homodimer of a polypeptide chain that displays a chemical fragmentation pattern, chromatographic behaviour and N-terminal amino-acid sequence identical to that of the PDGF A-chain[26].

We report here the complete primary structure of the PDGF A-chain precursor deduced from its complementary DNA sequence, its structural relation to the PDGF B-chain precursor, the chromosomal localization of the gene and its expression in human tumour cell lines. We also present data showing that the release of biologically active 31K PDGF-like growth factors by human tumour cell lines correlates with PDGF A-chain but not B-chain gene expression.

PDGF A-chain cDNA

A λgt10 cDNA library was constructed using poly(A)$^+$ RNA purified from the human clonal glioma cell line U-343 MGaC12:6. This cell line was chosen because it produces higher quantities of PDGF receptor competing activity than do other cell lines investigated. An 86-base-pair (bp) oligonucleotide probe (PDGF-A-1) corresponding to the N-terminus of the PDGF A-chain amino-acid sequence (Fig. 1) was synthesized and used to screen the library (2×10^6 recombinant clones) at low stringency. Of 48 positive clones, 4 hybridized to a 37-bp oligonucleotide probe (PDGF-A-2) directed against a mid-portion of the A-chain amino-acid sequence and were selected for further analysis.

DNA sequence analysis showed that the four clones overlapped and contained inserts of 800–1,400 bp (not shown). The complete nucleotide sequence, determined from one clone (D1), is shown in Fig. 1. The longest open reading frame of this 1.3-kilobase (kb) cDNA predicts a PDGF A-chain precursor protein of 211 amino acids (M_r ~23,000), and an in-frame termination codon is situated 81 bp upstream of the putative translation initiation site. Two additional ATG triplets lie within the 387 bp of the 5′-untranslated region sequenced, but these do not conform to the consensus for translation initiation[27] and predict only short polypeptides.

** Present addresses: Ludwig Institute for Cancer Research, Biomedicum, S-751 23 Uppsala, Sweden (C.-H.H.); KabiGen AB, Strandbergsgatan 49, S-112 87 Stockholm, Sweden (P.L.).

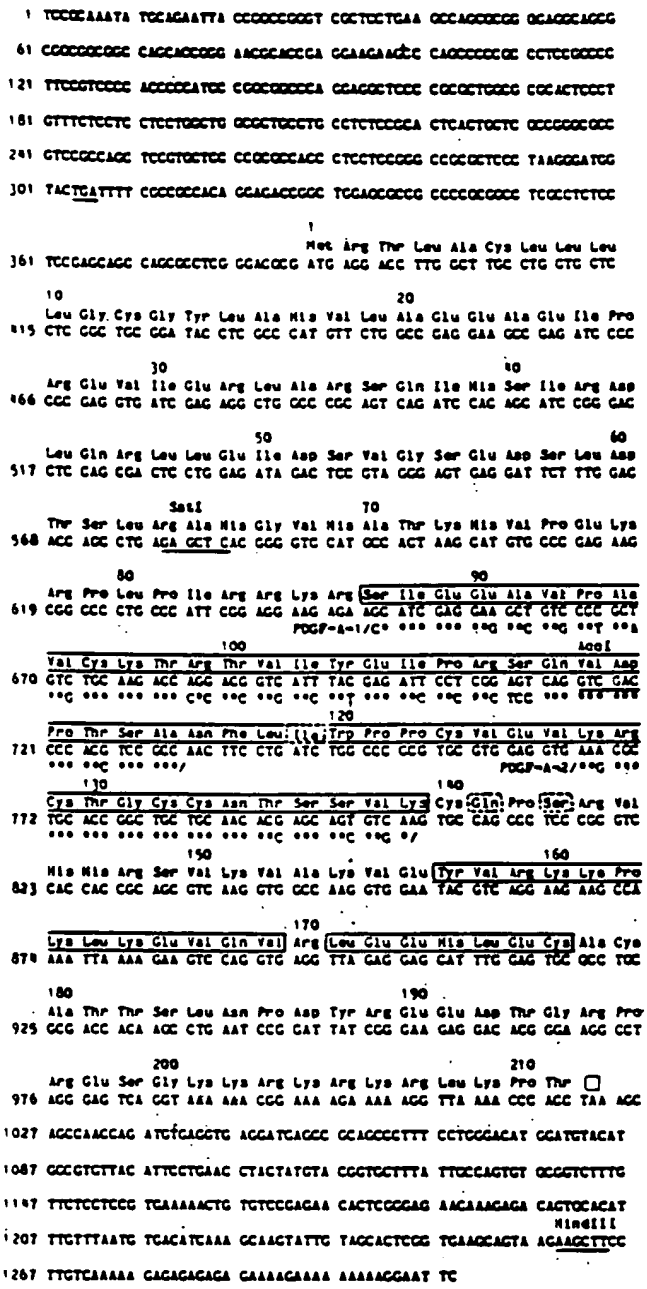

Fig. 1 Nucleotide sequence and deduced amino-acid sequence of the PDGF A-chain determined from a 1.3-kb cDNA clone (D1). An in-frame termination codon in the 5'-untranslated region is underlined. The PDGF A-chain cDNA encodes a 211-amino-acid precursor. Confirmed stretches of PDGF A-chain amino-acid sequence (from ref. 2) are boxed and differences indicated with dashed lines. Restriction endonuclease recognition sites used in the sequencing procedure are indicated. The sequences at which the two oligonucleotide probes PDGF-A-1 and PDGF-A-2 used to identify PDGF A-chain cDNAs were directed are indicated: * implies identity to the cDNA sequence. Box indicates termination codon.

Methods. Standard molecular biology techniques were used where not otherwise indicated. The double-stranded DNA probe PDGF-A-1 was synthesized as two overlapping 50-bp oligonucleotides and radiolabelled using [α-^{32}P]-deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I. PDGF-A-2 was synthesized as a 37-base template and a 12-base complementary primer and was radiolabelled as PDGF-A-1. Both oligonucleotides were synthesized using solid-phase phosphoramidite methodology[31]. The human clonal glioma cell line U-343.MGaC12:6 was the source of poly(A)$^+$ RNA, which was prepared using the LiCl/urea method modified as described elsewhere[11]. Oligo(dT)-primed synthesis of double-stranded cDNA was performed according to Gubler and Hoffman[32]. The resulting cDNA was treated with T4 DNA polymerase (Biolabs) and subcloned into *Eco*RI-cleaved λgt10 using *Eco*RI linkers. The recombinant phage were plated on *Escherichia coli* C600 hfl. Duplicate nitrocellulose filter lifts were hybridized with ^{32}P-labelled oligonucleotide probes at 42 °C in 20% formamide, 5 × SSC, 50 mM sodium phosphate *p*H 7.0, 5 × Denhardt's, 0.1% SDS, 200 μg ml^{-1} sonicated salmon sperm DNA and washed in 0.5 × SSC, 0.1% SDS at the same temperature. The nucleotide sequence of the PDGF A-chain cDNA restriction fragments was determined by dideoxynucleotide chain termination after subcloning into M13 phage derivatives.

The protein sequence matches that derived by amino-acid sequencing of the PDGF A-chain[2] except at amino acids 119, 141 and 143, found to be Ile, Gln and Ser, respectively, instead of the previously assigned Val, Arg and Thr (Fig. 1). These discrepancies could be due to protein sequencing errors. Alternatively, as the cDNA was obtained from a tumour cell line, it is possible that the sequence deviates from that of the normal PDGF A-chain transcript. The ATG codon at position 388 precedes a basic amino acid (Arg) followed by 18 hydrophobic residues (Fig. 1). This is characteristic of a signal peptide sequence and is consistent with the observation that PDGF A-chain homodimers produced by human osteosarcoma cells are secreted[25,26]. Comparison with preferred signal peptidase cleavage sites[28] suggests that processing may occur between amino acids Ala 20 and Glu 21. The N-terminal sequence of platelet PDGF A-chain is found at amino acid 87, indicating that a propeptide of 66 amino acids (44% charged residues) is cleaved from the precursor to generate a 125-amino-acid A-chain protein. This cleavage occurs after a run of four basic amino acids, Arg-Arg-Lys-Arg. Additional proteolytic processing may occur in the C-terminal region.

Human PDGF is heterogeneous in relative molecular mass, probably reflecting proteolytic cleavage in the platelets or degradation during the purification procedure. SDS-gel electrophoresis of the two constituent chains has revealed that the variability is confined mainly to the A-chain[1,4]. As amino-acid sequencing showed a unique A-chain N-terminus[2-4], this heterogeneity may arise through proteolysis in the highly basic C-terminus (Fig. 1). After N-terminal modification, the A-chain would have a M_r of ~14,000, although the highest-M_r form of the A-chain migrates as a 16–18K species on SDS gels[1,4]. The discrepancy may be due to glycosylation and/or the anomalous migration commonly observed for cationic proteins. A single consensus sequence for asparagine-linked glycosylation (Asn-X-Ser/Thr) is found at position 134–136, consistent with the report that PDGF contains carbohydrate[29]. The mature B-chain does not possess any *N*-glycosylation sites, although one is present in the N-terminal propeptide (Fig. 2).

The 5'-untranslated region of the PDGF A-chain messenger RNA has a high G+C content (~75%) and a high proportion of CpG dinucleotides. CpG-rich regions are found at the 5' end of many vertebrate genes and may indicate that these regions

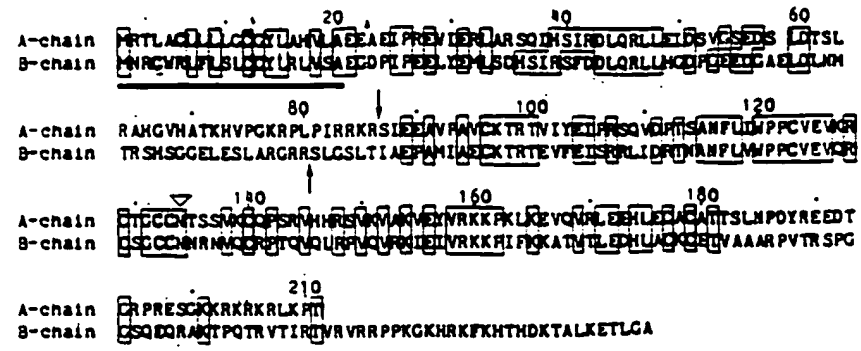

Fig. 2 Alignment and comparison of the two PDGF chain precursor amino-acid sequences. Homologies are boxed. Cysteine residues are shaded. Signal sequences are underlined and *N*-glycosylation sites are marked with a ▽. N-terminal processing sites are marked with arrows.

are protected from methylation[30]. Clone D1 carries 281 bp of 3'-untranslated sequence ending with a $(dGA)_6$ repeat followed by a short poly(A) stretch and *Eco*RI linker but no recognizable p lyadenylation signal. Of four cDNA clones sequenced, three terminate around this same position but a fourth contains a longer dGA repeat, extends 370 bp farther 3', but also lacks a polyadenylation signal and poly(A) tail (not shown). It is possible that the three similar clones, including D1, are primed internally on an oligo(A) stretch and represent a mRNA spliced differently from the clone with the longer 3' extension, a possibility in agreement with the presence of multiple A-chain transcripts (Fig. 3). The exact relationship between the different clones and mRNAs remains unknown, although cDNAs in which bases 968–1,036 (Fig. 1) are deleted have been identified (data not shown) and are believed to be the result of differential splicing. If translated, these clones predict an A-chain precursor 15 residues smaller and lacking the basic C-terminal region.

Relationship with the PDGF B-chain

Comparison of the amino-acid sequences of the PDGF A- and B-chain precursors shows them to be similar in size, with an overall amino-acid sequence homology of 40% after insertion of several gaps in their N-terminal portions. A significantly higher degree of homology is seen in a region within the mature chains; amino acids 89–181 of the A-chain is 56% homologous t the B-chain (Fig. 2). Notably, all eight cysteine residues are conserved within the mature chains, implying a similar tertiary structure. Accordingly, homodimers of either the B- or A-chain can bind to the PDGF receptor. The basic region Val-Arg-Lys-Lys-Pro (amino acids 158–162) may be relevant in this context, since basic polypeptides such as protamine sulphate and polylysine have been shown to compete with ^{125}I-PDGF for binding to the PDGF receptor[31].

A significant degree of homology is also seen between part of the N-terminal propeptide sequences, particularly a 10-amino-acid stretch at position 39–48 in the A-chain precursor. The analogous region in v-*sis* is not essential for this gene's transforming function[32]. In addition, apparently identical 24K B-chain dimers were formed in NIH 3T3 cells transfected with v-*sis* constructs with or without the N-terminal propeptide region[32]. Thus, it is difficult to assign a role for this regi n in post-translational processing of the two PDGF chains.

While there is essentially no sequence homology between the precursor C-terminal sequences, both contain a high proporti n of basic amino-acid residues (Fig. 2). Significant nucleotide sequence homology between the A- and B-chain transcripts is observed only in those regions where the amino-acid sequence is strongly conserved.

Hydrophobicity plots (data not shown) indicate that the A- and B-chain precursors are hydrophilic proteins with two maj r conserved hydrophobic domains. The first corresponds to the signal sequences, while the second is located 28 and 34 residues from the N-terminus of the processed A- and B-chain respectively (Fig. 2) and coincides with a 12-amino-acid conserved region in which there is only one difference (Ile/Val) between the two proteins.

Chromosomal localization

Using 36 human-mouse somatic cell hybrids, we mapped the PDGF A-chain gene to the pter→q22 region of chrom some 7 (Table 1). No other growth factor genes have been localized to this chromosome. The PDGF B-chain gene (c-*sis*) has been mapped[7] to the long arm of chromosome 22. Interestingly, after duplication of the ancestral PDGF gene, the A- and B-chain genes have acquired different chromosomal localizati ns.

PDGF mRNA expression in tumour cells

Northern blot hybridization analysis using poly(A)$^+$ RNA from various human cell types shows that the PDGF A-chain mRNA is expressed in several of the transformed cell lines examined but is not found in normal human fibroblasts or freshly is lated

Table 1 Distribution of the PDGF A-chain gene with human chromosomes in human-mouse cell hybrids

		Human chromosome																						
PDGF/Chrom.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
No. of concordant hybrids	(+/+)	9	12	13	12	11	10	19	12	5	16	13	13	12	16	12	8	17	12	9	12	15	8	10
	(−/−)	19	15	8	13	10	15	18	9	17	9	14	10	12	9	13	15	6	8	18	8	3	12	7
No. of discordant hybrids	(+/−)	9	7	7	8	9	10	0	8	15	4	6	7	8	4	7	12	3	8	11	8	5	11	6
	(−/+)	0	4	8	6	9	4	0	10	1	10	5	9	7	10	6	4	12	11	1	11	16	6	10
	% Discordancy	24	29	42	36	46	36	0	46	42	36	29	41	38	36	34	41	39	49	31	49	54	46	48

A PDGF A-chain cDNA probe (clone D1) was hybridized to Southern blots containing *Eco*RI- or *Hin*dIII-digested DNA from human-mouse hybrids. Presence of the human PDGF A-chain gene in the hybrids was determined by scoring the presence or absence of human bands on the blots. The first symbol in the parentheses indicates hybrids that were either positive (+) or negative (−) for the PDGF A-chain gene, while the second symbol indicates hybrids that either contained (+) or lacked (−) the particular chromosome. Concordant hybrids have either retained or lost the PDGF A-chain gene together with a specific human chromosome. Discordant hybrids either retained the gene, but not a specific chromosome, or the reverse. Per cent discordancy indicates the degree of discordant segregation of the PDGF A-chain gene and a chromosome. A 0% discordancy is the basis for chromosome assignment. One hybrid, JSR-17S, with a 7/9 translocation, indicates that the PDGF A-chain gene is localized to the pter→q22 region of chromosome 7. The table is compiled from 39 cell hybrids involving 14 unrelated human cell lines and 4 mouse cell lines[41,42]. The hybrids were characterized by chromosome analysis, by mapped enzyme markers and partly by mapped DNA probes.

Table 2 Compiled data on the expression of the PDGF A- and B-chain genes and secretion of PDGF-like growth factors by human tumour cell lines and normal cells

Cell line	B-chain mRNA	A-chain mRNA	Secretion of a 31K PDGF-like protein	PDGF-receptor competing activity (ng ml^{-1})	Mitogenic activity in conditioned medium inhibitable by PDGF antibodies
Tumour cells					
U-2 OS	+	++	++	10	+
U-4 SS	++	++	++	10	+
U-393 OS*	−	+	+	3	ND
SAOS-II	+	−	−	0	ND
SKLMS	−	+	+	2	ND
B-5 GT	−	++	++	15	+
B-6 FS	−	+	+	2	ND
RD	−	++	++	25	+
U-343 MGaC12:6	++	+++	+++	40	+
U-563 MG*	−	−	−	0	ND
Normal cells					
AG 1523	−	−	−	0	−
Macrophages	−	−	ND	ND	ND

The cell lines have the following origins: U-2 OS, osteosarcoma[43]; U-4 SS, synovial sarcoma[43]; U-393 OS, osteosarcoma; SAOS-II, osteosarcoma[44]; SKLMS, leiomyosarcoma[44]; B-5 GT, giant cell sarcoma[45]; B-6 FS, fibrosarcoma[45]; RD, rhabdomyosarcoma[46]; U-343 MGaC12:6, glioma[47]; U-563 MG, glioma; AG 1523, a human foreskin fibroblast line obtained from the Human Genetic Mutant Cell Repository, Camden, New Jersey. Macrophage RNA was prepared from freshly isolated peritoneal macrophages, collected by centrifugation of dialysis fluid (1,500*g*, 5 min). +/− Indicates presence/absence of hybridizing mRNAs on Northern blots or specifically immunoprecipitated 31K proteins that become converted to 16.5–17K species on reduction. PDGF receptor competing activity of serum-free tumour cell-conditioned medium was measured as inhibition of the binding of added ^{125}I-labelled PDGF to human foreskin fibroblasts[25,33,48]. Using a standard curve constructed from results obtained with pure unlabelled PDGF (5–200 ng ml^{-1}), PDGF receptor competing activity of the samples was converted to PDGF equivalents (ng ml^{-1}). Determination of mitogenic activity in serum-free tumour cell-conditioned medium was performed as described previously[49] in the absence or presence of 50 μg ml^{-1} of anti-PDGF IgG[50]. ND, not determined.

* Unpublished cell lines of Department of Pathology, Uppsala, Sweden.

peritoneal macrophages (Fig. 3). The macrophages we used were not activated *in vitro* before RNA preparation; after activation, macrophages have been found to express c-*sis* and produce a PDGF-like growth factor[14,15]. All positive cell lines display three major hybridizing bands, corresponding to transcripts of 1.9, 2.3 and 2.8 kb. Certain human tumour cell lines have been reported to express the PDGF B-chain (c-*sis*) transcript[8–11]. Some of the cell lines investigated here, such as the glioma U-343 MGaC12:6 and the osteosarcoma line U-2 OS, express both types of transcripts, whereas other cell lines, such as the rhabdomyosarcoma RD and the giant cell sarcoma B-5 GT, express only the A-chain mRNA, and the glioma U-563 MG, like normal fibroblasts and macrophages, expresses none. The A- and B-chain genes are thus regulated independently in human tumour cell lines examined.

Secretion of PDGF-like growth factors

The synthesis of PDGF-like growth factors by human tumour cell lines has been extensively reported[9–11,26,25,26,33–35]. These factors are all 31K proteins, split by reduction into two closely migrating 16.5K and 17K bands (Fig. 4)[10,25,33]. They possess the biological features of PDGF and are recognized by anti-PDGF antibodies. Our data show that immunoprecipitation of PDGF-like proteins from the conditioned medium of the human tumour cell lines studied correlates with the expression of PDGF A-chain but not B-chain mRNA (Table 2). This suggests that all of the PDGF-like fact rs detected by anti-PDGF antibodies in the medium f these human tum ur cell lines are c mp sed of only PDGF A-chains, despite the fact that s me express both A- and B-chain mRNA (Fig. 3). This view is supp rted by the detailed structural characterizati n of the 31K fact r secreted by U-2 OS cells which sh wed it to be an A-chain h modimer[26].

Discussion

Our study sh ws that the tw c nstituent chains f human PDGF are encoded by genes l cated n different chromosomes, and that both genes can be expressed independently in humar tumour cell lines.

PDGF is stored in the platelet α-granules and released ir conjunction with the platelet release reaction (reviewed in ref. 3) It is believed to act as a mitogen for connective tissue cells a the site of vascular injury. Homodimers of both PDGF A- anc B-chains possess PDGF receptor agonist activity. What, there fore, is the significance of the presence of both types of chain in human PDGF? Studies of the B-chain homodimer encodec by v-*sis* and the osteosarcoma-derived A-chain homodimer hav revealed differences in the efficiency of secretion and/or affinit

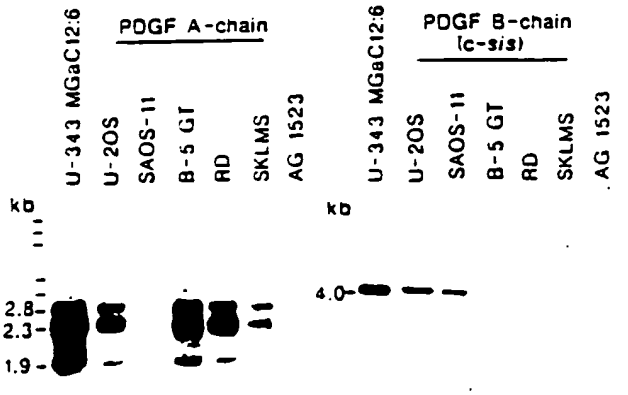

Fig. 3 Northern blot analysis of poly(A)$^+$ RNA (10 μg per lane) from various normal and neoplastic human cells. The origins of the cell lines are given in Table 2 legend. Cells growing in monolayer were collected at confluency. Total cellular RNA was prepared and selected once on oligo(dT)-cellulose (Pharmacia). Agarose gel electrophoresis, blotting to nitrocellulose and hybridization to ^{32}P-labelled PDGF A-chain cDNA (left) or PDGF B-chain (c-*sis*) cDNA (right) were performed as described previously[11]. Filters were exposed to Kodak XAR-5 films at −70 °C for 4 days.

Fig. 4 Immunoprecipitation of metabolically labelled PDGF-like growth factors produced by human tumour cells. The origins of the cell lines are given Table 2 legend. Confluent 350-cm² roller bottle cultures of cells were labelled with ^{35}S-cysteine (NEN, 600 Ci mmol^{-1}) as described elsewhere[23]. Briefly, cultures were pulsed with 250 μCi of ^{35}S-cysteine in 3 ml of cysteine-free medium for 3 h, and then chased in 3 ml of cysteine-containing medium for an additional 3 h. Media were pooled and sequentially precipitated with a control rabbit serum (*a*) and PDGF antiserum (*b*). Immunoprecipitates were absorbed to protein A-Sepharose (Pharmacia) and analysed on 13–18% SDS-polyacrylamide gels under reducing or non-reducing conditions. Dried gels were exposed to Kodak XAR-5 film for 4–7 days at −70 °C.

for a specific cellular compartment[23,36], raising the possibility that structural differences between the two chains serve different functions in relation to storage, release and association with the plasma membrane, extracellular matrix and plasma proteins[37–39]. For example, both types of homodimer are biologically active but their affinity for the PDGF receptor may differ both from each other and from the putative heterodimer. In fact, platelet PDGF appears to be more potent than the PDGF-like factors purified from human tumour cell line-conditioned media (C.-H.H. *et al.*, unpublished results). Furthermore, in spite of evidence that the transforming function of SSV is exerted by an externalized v-*sis* product, no accumulation of PDGF agonist activity is seen in the medium of acutely SSV-transformed human fibroblast cultures, and anti-PDGF antibodies precipitate only low-M_r monomers from the medium of SSV-transformed cells[23]. Apparently, after being released, the v-*sis* product remains associated with, or rapidly associates with, structures in the cell membrane including the PDGF receptor[18–36]. The low-M_r monomers probably represent degraded v-*sis* products. In contrast, intact 31K A-chain homodimers can be immunoprecipitated from human tumour cell-conditioned medium. The A-chain may therefore contribute to the stability of PDGF.

The exact nature of the human PDGF subunit composition and the significance of the presence of both A- and B-chains remain unknown. The genetic basis for A-chain expression in human tumour cells is also unknown, as is its role in tumour growth. Several non-transformed cell types, endothelial cells[12], cytotrophoblasts[13], smooth muscle cells[40] and activated macrophages[14,15] have been shown to express the c-*sis* gene and/or to release PDGF-like growth factors. It will be interesting to see whether the A-chain gene is expressed in these normal cells, and to determine the subunit structure of the secreted factors. Knowledge of the PDGF A-chain precursor structure and access to PDGF A-chain cDNAs as molecular probes will certainly contribute to the elucidation of such matters.

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